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Award Number: W81XWH-11-2-0006

TITLE: Rapid Dispersion of Polymicrobial Wound Biofilms with Depolymerase Enzymes

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REPORT DATE: November 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** | **2. REPORT TYPE** | **3. DATES COVERED** |
|---|---|---|
| 3 November 2012 | Annual | 4 October 2011 – 3 October 2012 |

**4. TITLE AND SUBTITLE**
Rapid Dispersion of Polymicrobial Wound Biofilms with Depolymerase Enzymes

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-11-2-0006

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Maryland
College Park, MD 20742-5100

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
During the performance period, we successfully developed protocols to extract biofilm EPS associated with ESKAPE organisms (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species). We further performed composition and linkage analysis on these samples and determined that 2 and 3-linked mannose residues constitute the major carbohydrate moiety in the EPS. In parallel, we synthesized, expressed, purified, and characterized 15 putative depolymerase enzymes by a variety of assays against static, dynamic, and mixed biofilm conditions. At least four enzymes (DSPB, NagZ, EQ, and Betty) will be advanced to safety and toxicity testing in the next performance period.

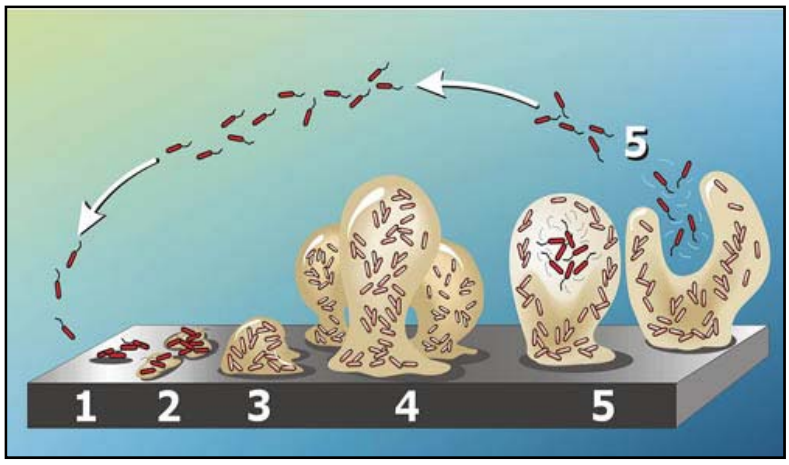
**15. SUBJECT TERMS**- Bacteriophage, depolymerase, biofilm, extrapolymeric substance, enzyme

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 25 | **19b. TELEPHONE NUMBER** *(include area code)* |

## Table of Contents

**INTRODUCTION:**

There is a critical need for basic research to discover new methods that would improve the outcomes of soldiers who incur battlefield wounds. Initially, most war wounds are colonized by Gram-positive bacteria. However, after initial stabilization and surgery, residual infections in open wounds are characterized by predominantly Gram-negative bacteria including *Acinetobacter baumannii-calcoaceticus* complex, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Additionally, wounded soldiers have a very high rate of hospital-acquired infections by such pathogens as *Staphylococcus aureus.* Thus, war wounds remain susceptible to infection from the time of injury through subsequent surgery, therapy, and rehabilitation and often contain multiple bacterial species. Moreover, each of these organisms has different resistance patterns to antibiotics, further limiting treatment options. Often, even broad-spectrum antibiotics are not sufficient to eradicate all of the organisms contained within a wound.




While bacterial pathogenesis mechanisms, virulence factors, and antimicrobial resistance vary greatly between pathogens associated with war wounds, one common trait shared by all is the ability to colonize wounds as a biofilm (see figure). Biofilms are formed when planktonic bacteria (i.e., free, individual cells) adsorb onto a wound surface and form multi-cellular colonies (figure, stage 1). Once the colonies become established, phenotypic changes cause them to secrete polysaccharides that serve as the backbone for the biofilm (figure, stage 2 and 3). Non-cellular components and debris, including additional carbohydrates, proteins, lipids, and nucleic acids, become entangled in the polysaccharide backbone and constitute the extrapolymeric substance (EPS) or "slime" layer of a biofilm (figure, stage 4). Significantly, the superstructure of the biofilm is known to protect internal bacteria from antimicrobials, antibodies, and circulating immune cells (figure, stage 5). *Thus, approaches that disrupt or dissolve the biofilm superstructure of polymicrobial infections would offer a therapeutic avenue to reduce the morbidity and mortality associated with war wounds by "re-sensitizing" the bacteria to antibiotics and the soldier's immune system.*

To accomplish this goal, we will use special enzymes called depolymerases. Depolymerases are normally found on the surface of bacterial viruses (i.e. bacteriophage) where they function to dissolve the EPS layer on naturally occurring biofilms allowing the phage to invade the bacterial cell. We plan to identify and test many such depolymerases to find the best enzyme, or cocktail of enzymes, that will dissolve biofilms associated with the bacteria that infect war wounds. Although the depolymerases do not directly kill the bacteria, it is believed that dissolution of the biofilm protective layer will allow common antibiotics or the immune system to clear the infection.

**BODY:**

Note: This is the second annual report for DM102823. The first annual report covered the following aims:

Aim 1. Task 1. IACUC review
Aim 1. Task 2. Bioinformatic analysis.
Aim 1. Task 3. Synthesize depolymerases.
Aim 1. Task 4. Obtain phage.
Aim 1. Task 5. Clone depolymerases.
Aim 1. Task 6. Express and purify depolymerases.
Aim 2. Task 1. Purify biofilm EPS.

This annual report covers the following aims:

Aim 2. Task 1. Purify biofilm EPS.
Aim 2. Task 2. Glycosyl composition.
Aim 2. Task 3. Glycosyl linkage.
Aim 3. Task 1. Static biofilms.
Aim 3. Task 2. Dynamic biofilms.
Aim 3. Task 3. Polymicrobial biofilms.

*Aim 2. Task 1. Purify biofilm EPS.*
*Aim 2. Task 2. Glycosyl composition.*
*Aim 2. Task 3. Glycosyl linkage.*

Since we selected strains that readily formed biofilms, establishing appropriate biofilm conditions was fairly simple. However, we spent considerable time developing and validating a novel method to extract the high molecular weight polysaccharide that constitutes the structural backbone of the biofilm EPS. Numerous protocols describe extraction methods for bacterial polysaccharide capsule as well as covalently attached surface polysaccharides, but very few are specific for biofilm EPS. Of those that are published, none allowed for the level of purification we required for our analysis. In the end, we took elements from three prior publications, combined with a gel filtration step in order to achieve the purity required. Below is a brief summary of our EPS polysaccharide purification flow chart.

Liu and Fang (2002) "Extraction of extracellular polymeric substances (EPS) of sludges". Journal of Biotechnology 95:249–256.

Oliveira, Marques, and Azeredo (1999) "Purification of polysaccharides from a biofilm matrix by selective precipitation of proteins". Biotechnology Techniques, 13:391-393.

Sofia Andersson (2009) "Characterization of bacterial biofilms for wastewater treatment". Ph.D. Thesis, Royal Institute of Technology, School of Biotechnology, Stockholm, Sweden

- Grow bacteria for several days to develop thick "sludge" biofilms

- Add 60 ul formaldehyde (36.5%) per 10 mls of "sludge" at 4C for 1 hour
- 4 mls 1N NaOH per 10 mls, 4C, 3 hours to extract bulk EPS from cells
- 20,000G centrifugation x 20 min, 4C
- Filter supernatant through 0.2 micron filter (may have to add DNAse to decrease viscosity from released nucleic acids).
- Dialyze against water 3500 MWCO membrane, 4C, 24 hours
- 20% TCA to precipitate protein as well as DNA, but leaves polysaccharides in the supernatant.
- 190-200 proof ethanol is used to precipitate polysaccharides away from lipids.
- Resuspend pellet and dialyze against water 3500 MWCO membrane, 4C, 24 hours
- Lyophilize overnight
- S-200 gel filtration to separate high molecular weight sugars from mono- and di-saccharides
- Phenol sulfuric acid assay to detect sugars in fractions
- Pool high molecular weight fractions.
- Dialyze against water 3500 MWCO membrane, 4C, 24 hours
- Lyophilize overnight.
- Samples ready for composition and linkage analysis.

The above protocol takes several weeks per sample. As a side note, since we had to develop such a novel extraction protocol that was not previously published, we are planning to submit it as a standalone protocol manuscript rather than as part of a larger manuscript.

The following samples were purified and were subjected to both composition and linkage analysis.

Sample ID: BAA1605
Source: EPS carbohydrate from *Acinetobacter baumannii*
04/10/12 ~7 mg

Sample ID: BAA1878
Source: EPS carbohydrate from *Acinetobacter baumannii*
04/10/12 ~5 mg

Sample ID: 700831
Source: EPS carbohydrate from *Klebsiella pneumoniae*
04/10/12 ~5 mg

Sample ID: 700603
Source: EPS carbohydrate from *Klebsiella pneumoniae*
04/10/12 ~23 mg

Sample ID: 700829
Source: EPS carbohydrate from *Pseudomonas aeroginosa*
04/10/12 ~9 mg

Sample ID: 700888
Source: EPS carbohydrate from *Pseudomonas aeroginosa*
04/10/12 ~5 mg

The completed composition analysis for six different strains shown below:

| 700829 (*P. aeruginosa*) | | |
|---|---|---|
| **Glycosyl residue** | **Mass (µg)** | **Mol %[1]** |
| Arabinose (Ara) | 3.7 | 1.6 |
| Rhamnose (Rha) | 2.7 | 1.0 |
| Fucose (Fuc) | 0.5 | 0.2 |
| Xylose (Xyl) | 1.0 | 0.4 |
| Galacturonic acid (GalA) | 2.1 | 0.7 |
| Mannose (Man) | 253.4 | 89.5 |
| Galactose (Gal) | 8.5 | 3.0 |
| Glucose (Glc) | 10.3 | 3.6 |
| Σ | 282.0 | 100.0 |

| BAA-1605 (*A. baumannii*) | | |
|---|---|---|
| **Glycosyl residue** | **Mass (µg)** | **Mol %[1]** |
| Arabinose (Ara) | 0.8 | 1.1 |
| Rhamnose (Rha) | 0.1 | 0.1 |
| Fucose (Fuc) | 0.1 | 0.1 |
| Xylose (Xyl) | 0.3 | 0.4 |
| Galacturonic acid (GalA) | 1.9 | 2 |
| Mannose (Man) | 73.9 | 84 |
| Galactose (Gal) | 3.5 | 4 |
| Glucose (Glc) | 7.0 | 7.9 |
| N-Acetyl Glucosamine (GlcNAc) | 0.5 | 0.4 |
| Σ | 88.0 | 100 |

| 700888 (*P. aeruginosa*) | | |
|---|---|---|
| **Glycosyl residue** | **Mass (µg)** | **Mol %[1]** |
| Arabinose (Ara) | 2.5 | 2.4 |
| Rhamnose (Rha) | 1.6 | 1.3 |
| Fucose (Fuc) | 0.3 | 0.2 |
| Xylose (Xyl) | 0.6 | 0.5 |
| Galacturonic acid (GalA) | 1.1 | 0.8 |
| Mannose (Man) | 109.2 | 84.3 |
| Galactose (Gal) | 4.8 | 3.7 |
| Glucose (Glc) | 8.8 | 6.8 |
| Σ | 128.8 | 100 |

| BAA-1878 (*A. baumannii*) | | |
|---|---|---|
| **Glycosyl residue** | **Mass (µg)** | **Mol %[1]** |
| Arabinose (Ara) | 1.0 | 0.5 |
| Rhamnose (Rha) | 0.1 | 0 |
| Xylose (Xyl) | 0.3 | 0.2 |
| Galacturonic acid (GalA) | 2.5 | 1 |
| Mannose (Man) | 181.2 | 79.3 |
| Galactose (Gal) | 30.3 | 13.2 |
| Glucose (Glc) | 10.3 | 4.5 |
| N-Acetyl Glucosamine (GlcNAc) | 3.5 | 1.3 |
| Σ | 229.1 | 100 |

| 700831 (*K. pneumonia*) | | |
|---|---|---|
| **Glycosyl residue** | **Mass (µg)** | **Mol %[1]** |
| Arabinose (Ara) | 2.3 | 1.5 |
| Rhamnose (Rha) | 16.3 | 10 |
| Fucose (Fuc) | 0.4 | 0.3 |
| Xylose (Xyl) | 0.7 | 0.5 |
| Galacturonic acid (GalA) | 18.9 | 9.8 |
| Mannose (Man) | 68.4 | 38.3 |
| Galactose (Gal) | 14.0 | 7.8 |
| Glucose (Glc) | 55.5 | 31.1 |
| N-Acetyl Glucosamine (GlcNAc) | 1.5 | 0.7 |
| Σ | 178.0 | 100 |

| 700603 (*K. pneumoniae*) | | |
|---|---|---|
| **Glycosyl residue** | **Mass (µg)** | **Mol %[1]** |
| Rhamnose (Rha) | 54.8 | 27 |
| Glucuronic Acid (GlcA) | 11.4 | 5 |
| Galacturonic acid (GalA) | 6.1 | 2.5 |
| Mannose (Man) | 110.8 | 49.4 |
| Galactose (Gal) | 32.4 | 14.4 |
| Glucose (Glc) | 2.8 | 1.3 |
| N-Acetyl Glucosamine (GlcNAc) | 2.1 | 0.8 |
| Σ | 220.5 | 99.9 |

**Linkage analysis**

For glycosyl linkage analysis, the sample was permethylated, depolymerized, reduced, and acetylated; and the resulting partially methylated alditol acetates (PMAAs) analyzed by gas chromatography-mass spectrometry (GC-MS) as described by York *et al* (1985) *Methods Enzymol.* 118:3-40.

Initially, dry sample was suspended in about 300 ul of dimethyl sulfoxide and placed on a magnetic stirrer for 5 days. The sample was then permethylated by the method of Ciukanu and Kerek (1984) *Carbohydr. Res.* 131:209. The sample was subjected to the NaOH base then methyl iodide was added. The base and more methyl iodide was added again. This addition of more methyl iodide and NaOH base was to insure complete methylation of the polymer. Following sample workup, the permethylated material was hydrolyzed using 2 M trifluoroacetic acid, reduced with $NaBD_4$, and acetylated using acetic anhydride/trifluoroacetic acid. The resulting PMAAs were analyzed on a Hewlett Packard 5975C GC interfaced to a 7890A MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30m Supelco 2330 bonded phase fused silica capillary column.

**Note:** The resulted spectrum showed 2-linked mannopyranosyl residue and 3-linked mannopyranosyl residues eluting together. Therefore we analyzed the sample again in an Agilent 6890N GC interfaced to a 5975B MSD, using a Supelco EC-1 fused silica capillary column (30m × 0.25 mm ID) to be able to distinguish these two residues. The ratio of 2-mannose and 3-mannose were thus determined from the resulting spectra and the percentage of peak area corresponding to the linkage analysis was calculated.

## Results:

### 700603

| GLYCOSYL RES | PK AREA% |
|---|---|
| terminally linked rhamnopyranosyl residues (t-man) | 1.6 |
| 2-linked rhamnopyranosyl residue (2-Rha) | 7.3 |
| terminally linked mannopyranosyl residues (t-man) | 1.3 |
| terminal linked galactopyranosyl residue (t-gal) | 0.3 |
| 2-linkedmannopyranosyl (2-Man) | 40.1 |
| 3-linked mannopyranosylresidue (3-Man) | 15.4 |
| 3-linked galactopyranosyl residue (3-Gal) | 18.2 |
| 4-linked glucopyranosyl residue (4-Glc) | 0.4 |
| 2,3-linked mannopyranosyl residue (2,3-man) | 15.4 |

### 700829

| GLYCOSYL RES | Peak Area (%) |
|---|---|
| terminally linked mannopyranosyl residues (t-man) | 23.3 |
| terminal linked galactopyranosyl residue (t-gal) | 0.1 |
| 4 linked arabinopyranosyl residue (4-Ara) | 0.2 |
| 3-linked glucopyranosyl residue (3-Glc) | 1.2 |
| 2-linkedmannopyranosyl (2-Man) | 20.6 |
| 3-linked mannopyranosylresidue (3-Man) | 16.7 |
| 6-linked mannopyranosyl residue (6-Man) | 1.4 |
| 6-linked glucopyranosyl residue (6-Glc) | 0.6 |

| | |
|---|---|
| 4-linked galactopyranosyl residue (4-Gal) | 1.2 |
| 4-linked glucopyranosyl residue (4-Glc) | 0.2 |
| 2,3-linked mannopyranosyl residue (2,3-man) | 1.3 |
| 3,6-linked mannopyranosyl residue (3,6-Man) | 0.6 |
| 2,6-linked mannopyranosyl residue (2,6-man) | 32.3 |
| 3,6-linked galacopyranosyl residue (3,6-Gal) | 0.3 |

## 700831

| **GLYCOSYL RES** | PK AREA% |
|---|---|
| terminally linked rhamnopyranosyl residues (t-Rha) | 0.3 |
| terminally linked mannopyranosyl residues (t-man) | 10.5 |
| terminallylinked glucopyranosylresidue (t-Glc) | 1.2 |
| 4 linked arabinopyranosyl residue (4-Ara) | 0.2 |
| 3-linked glucopyranosyl residue (3-Glc) | 14.4 |
| 2-linkedmannopyranosyl (2-Man) | 13.8 |
| 3-linked mannopyranosylresidue (3-Man) | 5.2 |
| 2-linked hexafuranosyl residue (2-hexf) | 12.2 |
| 6-linked mannopyranosyl residue (6-Man) | 1.2 |
| 6-linked glucopyranosyl residue (6-Glc) | 0.4 |
| 4-linked galactopyranosyl residue (4-Gal) | 0.2 |
| 4-linked glucopyranosyl residue (4-Glc) | 0.4 |
| 2,3-linked mannopyranosyl residue (2,3-man) | 0.5 |
| 2,3,4-linked mannopyranosyl residue (2,3-man) | 11.6 |
| 3,6-linked mannopyranosyl residue (3,6-Man) | 0.4 |

| | |
|---|---|
| 2,6-linked mannopyranosyl residue (2,6-man) | 16.1 |
| 4,6-linked glucopyranosyl residue (4,6-Glc) | 11.4 |

## 700888

| **GLYCOSYL RES** | PK AREA% |
|---|---|
| terminally linked mannopyranosyl residues (t-man) | 27.4 |
| terminal linked galactopyranosyl residue (t-gal) | 0.2 |
| 4 linked arabinopyranosyl residue (4-Ara) | 0.2 |
| 2-linked xylopyranosyl residue (2-Xyl) | 0.1 |
| 3-linked glucopyranosyl residue (3-Glc) | 1.3 |
| 2-linkedmannopyranosyl (2-Man) | 19.3 |
| 3-linked mannopyranosylresidue (3-Man) | 16.0 |
| 3-linked galactopyranosyl residue (3-Gal) | 0.3 |
| 4-linked mannopyranosyl residue (4-Man) | 0.4 |
| 6-linked mannopyranosyl residue (6-Man) | 1.6 |
| 6-linked glucopyranosyl residue (6-Glc) | 0.7 |
| 4-linked galactopyranosyl residue (4-Gal) | 1.6 |
| 4-linked glucopyranosyl residue (4-Glc) | 0.4 |
| 2,3-linked mannopyranosyl residue (2,3-man) | 1.2 |
| 3,6-linked mannopyranosyl residue (3,6-Man) | 0.7 |
| 2,6-linked mannopyranosyl residue (2,6-man) | 28.5 |
| 3,6-linked galacopyranosyl residue (3,6-Gal) | 0.2 |

## BAA1605

| **GLYCOSYL RES** | **PK AREA%** |
|---|---|
| terminally linked mannopyranosyl residues (t-man) | 25.4 |
| 2-linkedmannopyranosyl (2-Man) | 20.2 |
| 3-linked mannopyranosylresidue (3-Man) | 16.5 |
| 6-linked mannopyranosyl residue (6-Man) | 2.2 |
| 6-linked glucopyranosyl residue (6-Glc) | 1.3 |
| 4-linked galactopyranosyl residue (4-Gal) | 1.1 |
| 3,6-linked galacopyranosyl residue (3,6-Gal) | 0.8 |
| 2,6-linked mannopyranosyl residue (2,6-man) | 32.5 |

## BAA1878

| **GLYCOSYL RES** | PK AREA% |
|---|---|
| terminally linked mannopyranosyl residues (t-man) | 27.4 |
| terminal linked galactopyranosyl residue (t-gal) | 0.2 |
| 4 linked arabinopyranosyl residue (4-Ara) | 0.2 |
| 2-linked xylopyranosyl residue (2-Xyl) | 0.1 |
| 3-linked glucopyranosyl residue (3-Glc) | 1.3 |
| 2-linkedmannopyranosyl (2-Man) | 19.2 |
| 3-linked mannopyranosylresidue (3-Man) | 15.8 |
| 3-linked galactopyranosyl residue (3-Gal) | 0.3 |
| 4-linked mannopyranosyl residue (4-Man) | 0.4 |
| 6-linked mannopyranosyl residue (6-Man) | 1.6 |
| 6-linked glucopyranosyl residue (6-Glc) | 0.7 |

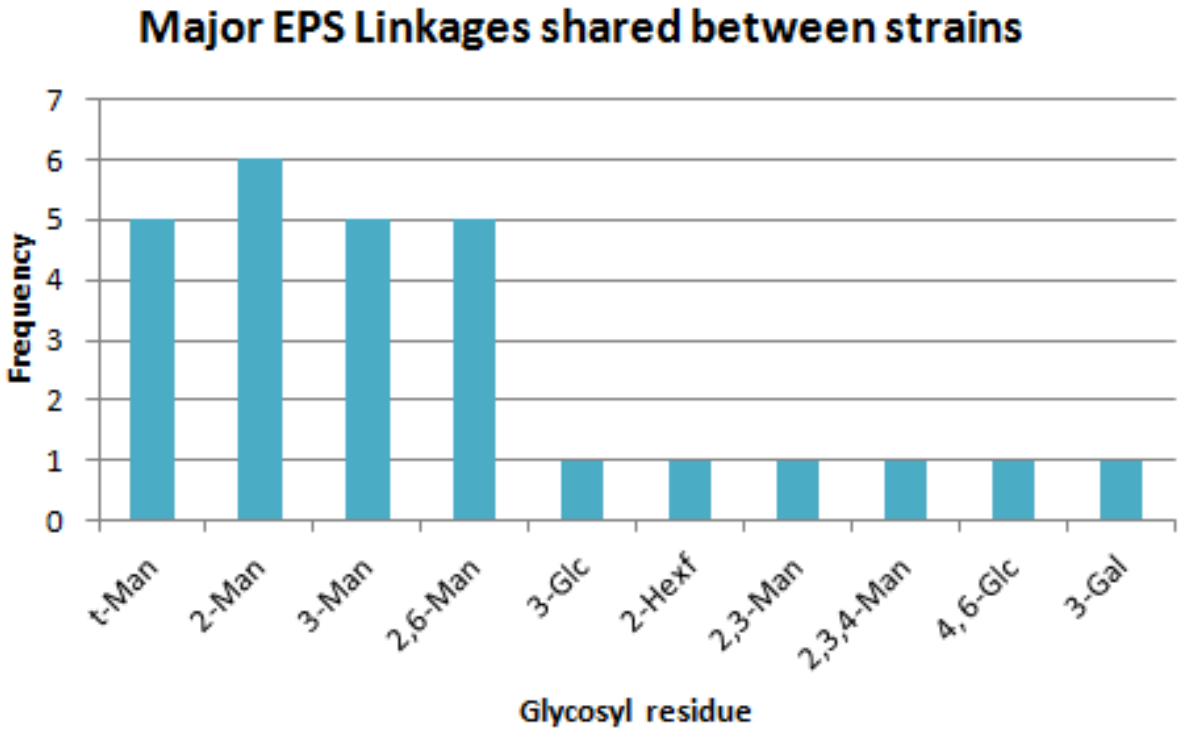
| | |
|---|---|
| 4-linked galactopyranosyl residue (4-Gal) | 1.6 |
| 4-linked glucopyranosyl residue (4-Glc) | 0.4 |
| 2,3-linked mannopyranosyl residue (2,3-man) | 1.2 |
| 3,6-linked glucopyranosyl residue (3,6-Glc) | 0.7 |
| 2,6-linked mannopyranosyl residue (2,6-man) | 28.5 |
| 3,6-linked galacopyranosyl residue (3,6-Gal) | 0.2 |

**Comments:**

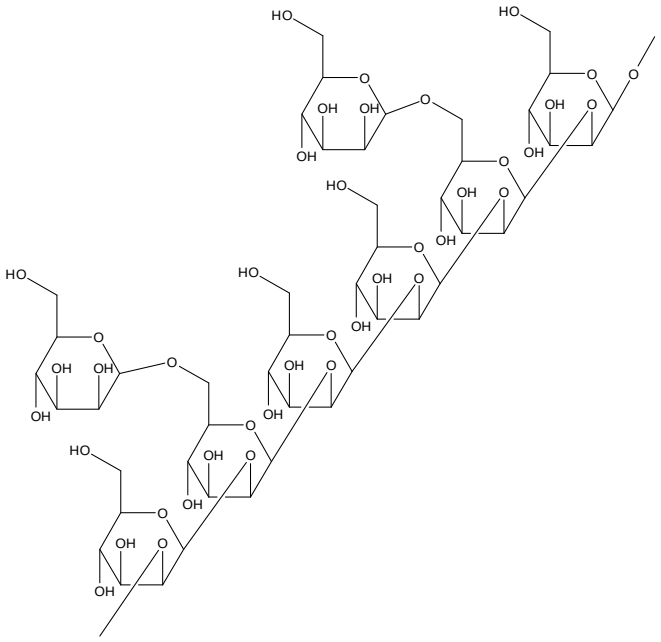
Linkage results indicate that all samples mainly consist of terminally linked mannopyranosyl residue (t-man), 2-linked mannopyranosyl (2-Man) and 3-linked mannopyranosyl residue (3-Man). 2,6-linked mannopyranosyl residue (2,6-man) were found in all samples except **700603**. Other linkage residues of galactose, glucose and mannose were also found in all samples. Small amount of terminally linked rhamnopyranosyl residue (t-Rha), 2-linked rhamnopyranosyl residue (2-Rha) were found in sample **700603**. 2-linked hexafuranosyl residue (2-hexf) was only found in sample **700831**. 4-linked arabinopyranosyl residue (4-Ara) was found in **BAA1878, 700831, 700888 and 700829**. 2-linked xylopyranosyl residue (2-Xyl) was only found in sample **700888**.

Surprisingly, all biofilms contained a very high content of mannose. In addition to composition analysis above, linkage analysis of the glycosyl units was also performed by GC/MS on permethylated, depolymerized, reduced, and acetylated EPS samples. As expected, a high percentage of all EPS linkages were found to include mannose. Linkages that composed 10% or more of all linkages of at least one EPS sample are given in the table below. (PA is *Pseudomonas aeruginosa*, KP is *Klebsiella pneumonia*, and AB is *Acinetobacter baumannii*).

| Glycosyl residue | Peak Area (%) | | | | | |
|---|---|---|---|---|---|---|
| | PA | | KP | | AB | |
| | 700829 | 700888 | 700603 | 700831 | 1605 | 1878 |
| t-Man | 23.3 | 27.4 | 1.3 | 10.5 | 25.4 | 27.4 |
| 2-Man | 20.6 | 19.3 | 40.1 | 13.8 | 20.2 | 19.2 |
| 3-Man | 16.7 | 16 | 15.4 | 5.2 | 16.5 | 15.8 |
| 2,6-Man | 32.3 | 28.5 | N/A | 16.1 | 32.5 | 28.5 |
| 3-Glc | N/A | N/A | N/A | 14.4 | N/A | N/A |
| 2-Hexf | N/A | N/A | N/A | 12.2 | N/A | N/A |
| 2,3-Man | N/A | N/A | 15.4 | N/A | N/A | N/A |
| 2,3,4-Man | N/A | N/A | N/A | 11.6 | N/A | N/A |
| 4, 6-Glc | N/A | N/A | N/A | 11.4 | N/A | N/A |
| 3-Gal | N/A | N/A | 18.2 | N/A | N/A | N/A |



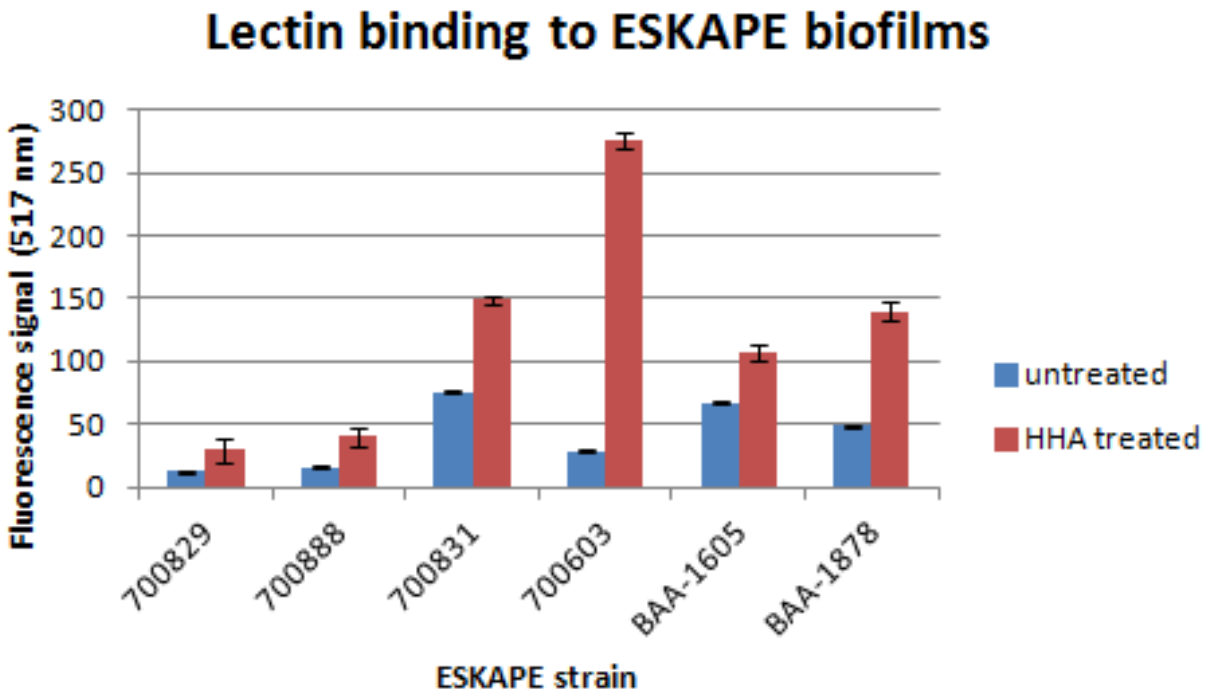
A high percentage of 1-2 linked mannose was found in all 6 tested ESKAPE strains' EPS



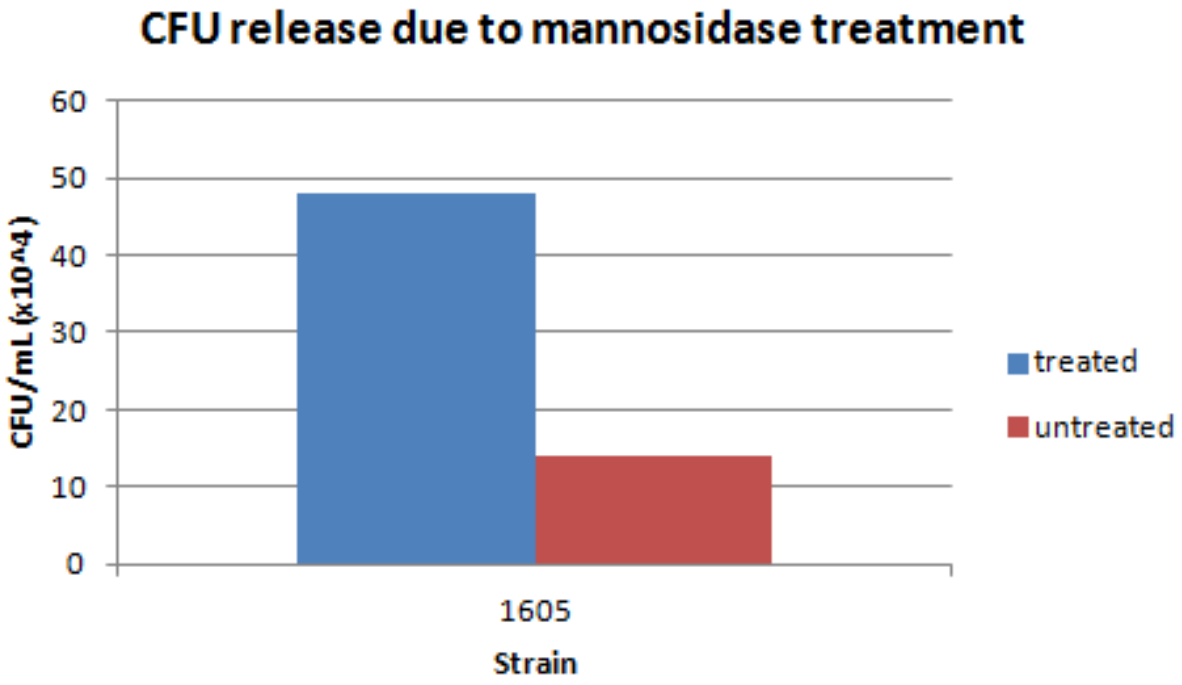
Linkage analysis data can be used to get a rough idea of the overall structure of a particular exopolysaccharide. Above is a representation of the likely structure of sample 700826 (mass spectrometry would be necessary confirm the exact structure).

Confirmation of high mannose content was achieved by assaying lectin-biofilm binding. Since all strains contained a high proportion of mannose 1-3 or mannose 2-6 linkages, the fluorescently-labeled lectin HHA (provided by EY Laboratories), was chosen. HHA binds specifically to α1-3 and α1-6 mannosyl residues and likely α2-6 residues as well (our data).

Biofilms were grown for 4 days in 96-well plates, washed 3 times with PBS, incubated with 10 ug HHA for 15 min, and then washed 3 more times. Fluorescence at 517 nm was observed using an excitation wavelength of 472 nm. When necessary, normalization to account for the amount of biofilm development was done using crystal violet staining.




Because many biofilms possess α1-2 linked mannose residues, we purchased a commercial mannosidase α1-2 mannosidase from New England Biolabs and evaluated its anti-biofilm properties. Preliminary data (figure, next page) suggests that this enzyme cleaves man α1-2 linkages may disperse biofilms of sample 1605 according to a bacterial CFU release assay. In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the supernatant is serial diluted and plated to enumerate bacterial colony forming units (CFU) present. An active enzyme will digest the biofilm matrix and increase the CFUs released to the supernatant. Research into the reproducibility of this result as well as the effect of mannosidases on other ESKAPE biofilms' dispersal is ongoing.




*Aim 3. Task 1. Static biofilms.*
*Aim 3. Task 2. Dynamic biofilms.*
*Aim 3. Task 3. Polymicrobial biofilms.*

In parallel with the characterization of the EPS biofilm of Aim 2, we continued to develop and evaluate our top depolymerase enzymes on static, dynamic, and polymicrobial biofilms (Aim 3). Currently, our top depolymerases are DSP, SF6, and CBA_120, however, we do have more in the pipeline that have just recently been characterized. For these experiments, we utilized three different methods to demonstrate depolymerase activity. For all methods, we first grew static biofilms in 96 well plates for 24 hours. Examples of data collected from each method and a summary chart of our current findings is below.

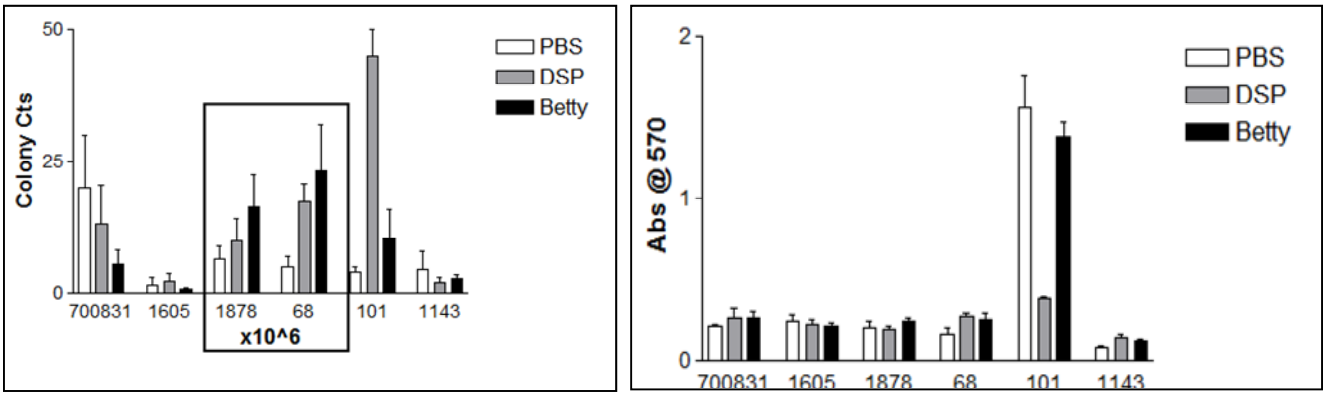
1. Biomass Eradication Assay. In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the remaining biofilm biomass is stained with 0.1% crystal violet. The crystal violet can be quantified by extraction in 1% SDS and read on a spectrophotometer at 590 nm. An active enzyme will decrease the crystal violet staining compared to controls.

2. Bacterial CFU Release Assay. In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the supernatant is serial diluted and

plated to enumerate bacterial colony forming units (CFU) present. An active enzyme will digest the biofilm matrix and increase the CFUs released to the supernatant.

3. Carbohydrate Release Assay. In this assay, biofilm wells are treated with putative depolymerase enzymes or buffer, incubated, and the supernatant is assayed for total carbohydrate content by the phenol sulfuric acid method (Dubois, M., K. A., Gilles, J. K. Hamilton, P. A., Rebers and F. Smith, Anal. Chem. 28, 350 (1956)). An active enzyme will digest the biofilm matrix and increase total carbohydrate content of the supernatant.

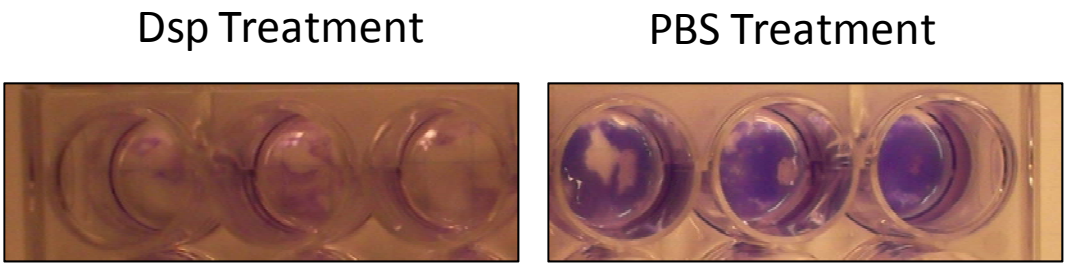
Below is just one sample of data against static biofilms:




| | |
|---|---|
| 700831 | known biofilm producer of *Klebsiella pneumonia* |
| 1605 | multi-drug resistant isolate of *Acinetobacter baumanii* from Afghanistan |
| 1878 | *Acinetobacter baumanii* from a burn patient |
| 68 | Enteric organism with expanded spectrum beta-lactamase resistance (i.e., ESBL) |
| 101 | Methicillin-resistant *Staphylococcus aureus* (i.e., MRSA) |
| 1143 | Enterobacteria with AmpC mediated ESBL |

The panel on the left depicts bacterial counts in the supernatants of static biofilms treated with PBS, or two depolymerases, DSP and Betty. If the depolymerases break down the biofilm, we would expect to see an increase in colony counts of the biofilm supernatant, which we see for *Acinetobacter baumanni*, an Enteric organism, and a MRSA strain. The panel on the right depicts a similar test of static biofilm bacteria, except we stained the remaining biofilm with crystal violet and measured the amount of residual biomass (which intercalates crystal violet in the biofilm). As can be seen, the depolymerases work well with the MRSA biofilm, but the

results are not as clear cut with the other biofilms which do not appear to take us as much crystal violet in the control wells. A picture of the actual MRSA wells is below to illustrate the anti-biofilm properties of Dsp.




Thus it appears that supernatant colony counts are more quantitative of depolymerase activity than crystal violet, which displays wide variation in staining properties between biofilms of different bacterial species.

Below is a chart that summarizes our finding for DSP, SF6, and CBA_120 from the past several months:

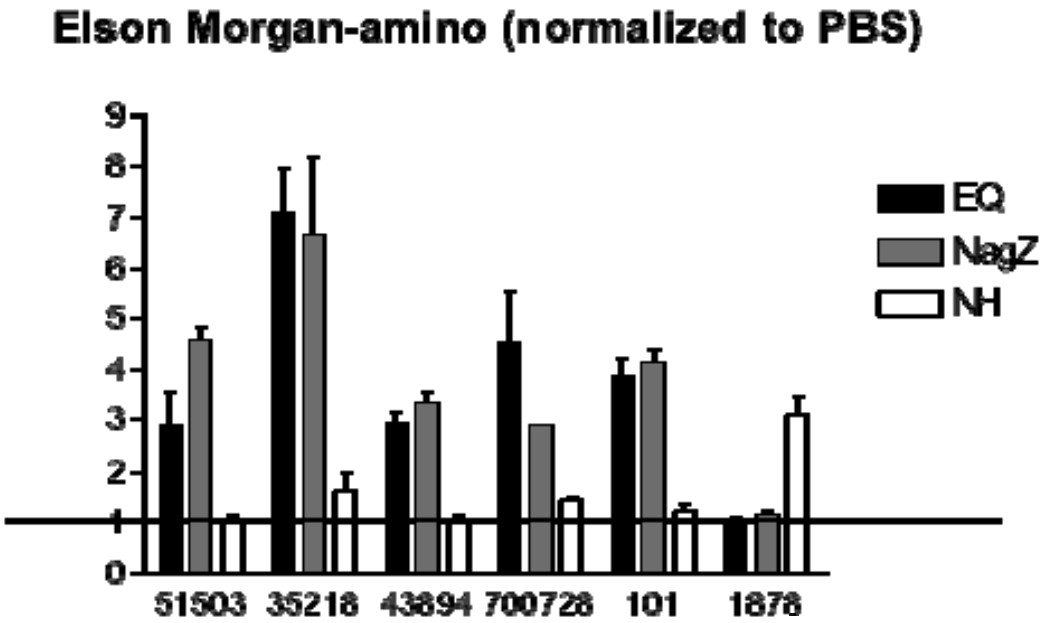
| ATCC # | Organism | Biomass Reduction | Bacterial CFU Release | Carbohydrate Reslease |
|---|---|---|---|---|
| 1605 | *Acinetobacter baumannii* | | DSP | DSP |
| 1878 | *Acinetobacter baumannii* | | DSP/CBA_120/SF6 | |
| 35218 | *E.Coli* | | DSP/CBA_120 | |
| 700728 | *E. coli O157:H7* | | CBA_120/SF6 | DSP/CBA_120/SF6 |
| 43894 | *E. coli O157:H7* | | | DSP/CBA_120/SF6 |
| 1143 | *Enterobacter* | | DSP | DSP/CBA_120 |
| 68 | *Enterobacter* | | DSP/CBA_120 | CBA_120 |
| 700603 | *Klebsiella pneumonia* | | DSP/CBA_120 | DSP/CBA_120/SF6 |
| 10145 | *Pseudomonas aeruginosa* | | DSP/CBA_120 | |
| 27853 | *Pseudomonas aeruginosa* | DSP/CBA_120/SF6 | DSP/Betty/SF6 | |
| 700829 | *Pseudomonas aeruginosa* | | CBA_120 | |
| 101 | *Staphylococcus aureus* | DSP/CBA_120 | DSP/CBA_120 | |

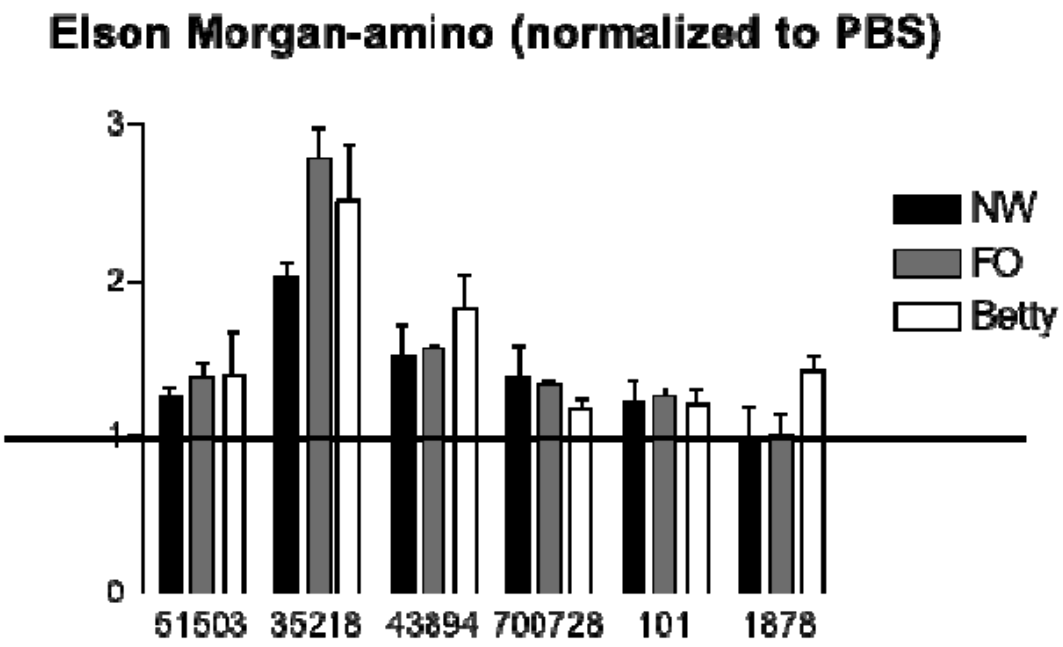
Our proposal called for evaluation of 15-20 potential depolymerases overall. However, we did not start out with this number. Instead, we only evaluated a couple so we could select future depolymerases based on knowledge gained from the first characterized depolymerases (i.e., it is an iterative process). In our early reports, we were quite keen on tail spike proteins for their putative depolymerase activity. However, we found this class of protein to be very species-specific and only possessed a low level of depolymerase activity. Recent enzymes added to the

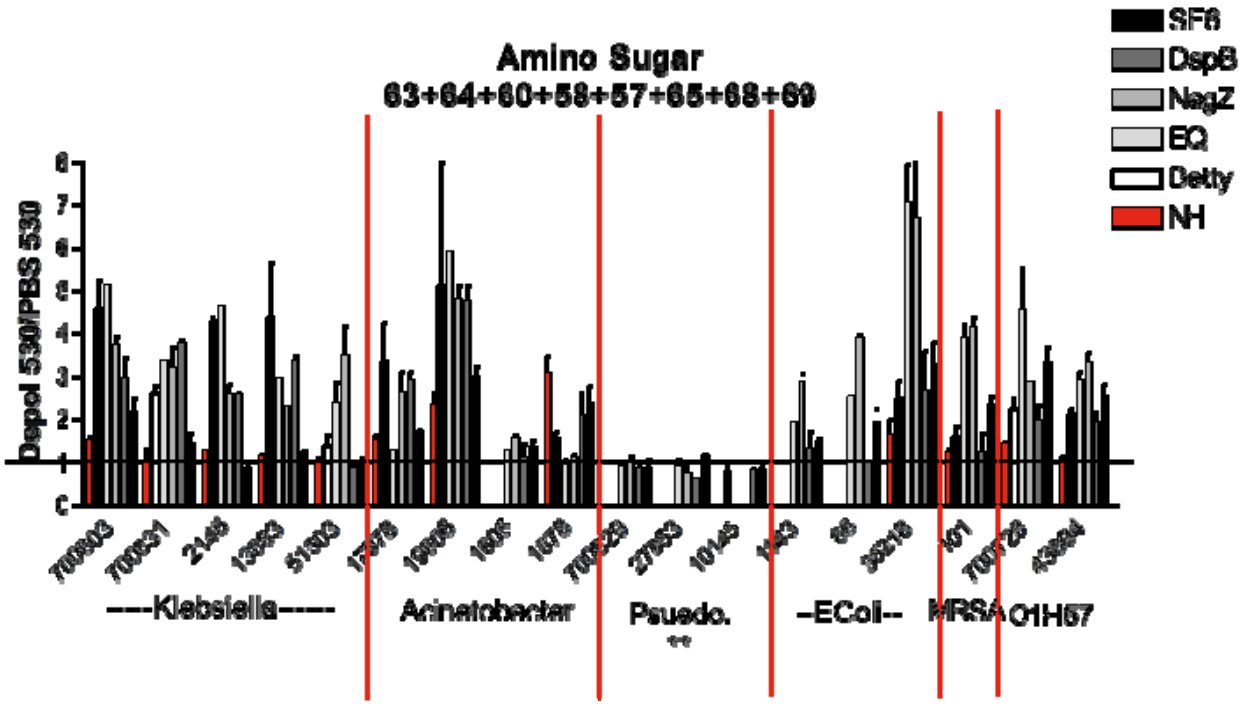
list include classes of lyases and glucronidases, which correspond to major bonds found in biofilm EPS. Below is a chart summarizing our current progress. 15 putative depolymerases have been synthesized, 11 have been cloned and transformed into expression systems, and 8 have been purified and partially characterized. The lead candidates will be assessed in safety and toxicity testing (Q9-Q12).

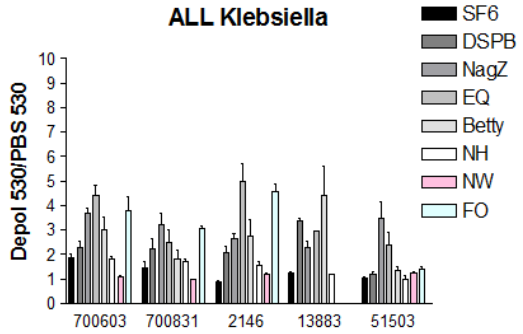
| GeneArt name | Characteristics | Target EPS | Predicted MW (kDa) | Cloning into pBAD24 | Transformation into BL21(DE3) | Expression/ Characterization |
|---|---|---|---|---|---|---|
| Dispersin B | Known depolymerase, active against Staph spp. and E. coli | Staph spp., E. coli | 37 | Y | Y | Y |
| SL-DspB | Staphylococcal homolog of Dispersin B | Staph spp., E. coli | 35 | Y | Y | Y |
| SF6 | Known tailspike | E. coli | 65.8 | Y | Y | Y |
| CPA_120_160c | Putative CPA_120 myophage tail-spike | E. coli O157:H7 | 81.96 | Y | Y | Y |
| DSP-NW | Related to DspB, from Neisseria sp. | Staph spp., E. coli | 57.9 | Y | Y | Y |
| DSP-AA | Related to DspB, from Aggregatibacter sp. | Staph spp., E. coli | 46.7 | Y | Y | Y |
| Lyase-U | Related to Klebsiella alginate lyase, from uncultured | P. aeruginosa | 36.2 | Y | Y | Y |
| Lyase-SD | Related to Klebsiella alginate lyase, from S. degradans | P. aeruginosa | 57.1 | Y | Y | Y |
| Lyase-MB | Microbulbifer protein related to Klebsiella alg lyase | P. aeruginosa | 39 | Y | Y | TBD |
| Lyase-PA | Related to Klebsiella alginate lyase, from P. aeruginosa | P. aeruginosa | 41.6 | Y | Y | TBD |
| Lyase-SM | Related to Pseudomonas alginate lyase, from S. maltophilia | P. aeruginosa | 37.7 | Y | Y | TBD |
| Gluc-Eq | Related to E. coli glucuronidase, from S. equi | K. pneumonia | 69.2 | TBD | TBD | TBD |
| Gluc-NH | Related to E. coli glucuronidase, from N. haematocca | K. pneumonia | 68.9 | TBD | TBD | TBD |
| Gluc-FO | Related to E. coli glucuronidase, from F. oxysporum | K. pneumonia | 69.1 | TBD | TBD | TBD |
| NagZ-AB | Related to E. coli NagZ, sequenced from Acinetobacter | Acinetobacter baumanii | 39 | TBD | TBD | TBD |

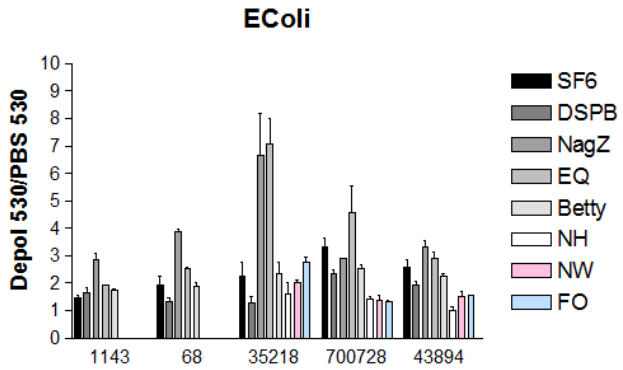
We have performed dozens and dozens of experiments with the above enzymes in static, dynamic, and mixed biofilms using the biomass eradication assay, the bacterial CFU release assay, and the carbohydrate release assay, also known as the Elson Morgan assay. We have concluded that the carbohydrate release (Elson Morgan) assay gives us the most reproducible results for depolymerase enzymatic activity. Below is just a brief sample of our results with some of the enzymes listed above. Clearly, different enzymes display efficacy for certain ESKAPE strains. The best enzymes will be forwarded to safety and toxicity testing in Q9-Q12.

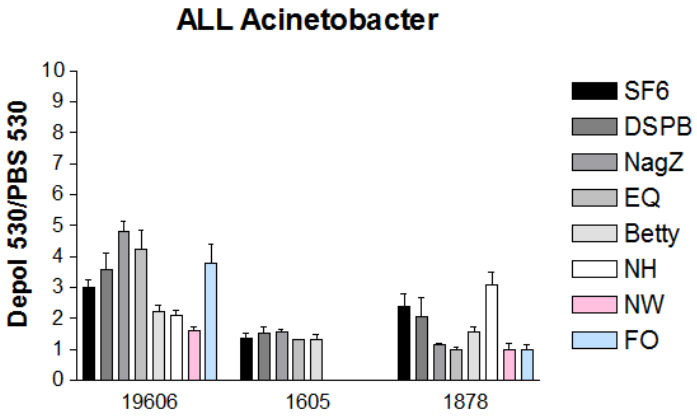



Elson Morgan-amino (normalized to PBS)
3
2
1
0
NW
FO
Betty
51503
35218
43894
700728
101
1878


Amino Sugar
63+64+60+58+57+65+68+69
SF6
DspB
NagZ
EQ
Betty
NH
Dispol 530/PBS 530
---Klebsiella-----
Acinetobacter
Psuedo.
--EColi--
MRSA
O1H57




## KEY RESEARCH ACCOMPLISHMENTS:

- Extracted EPS from biofilms of ESKAPE organisms
- Performed composition analysis for EPS from ESKAPE organisms
- Performed linkage analysis for EPS from ESKAPE organisms
- Successfully synthesized, expressed, and purified and evaluated ~15 putative depolymerase enzymes
- Assayed depolymerase enzymes in static, dynamic, and polymicrobial biofilms

## REPORTABLE OUTCOMES:

Some aspects of our preliminary data and/or overall strategy have been presented at the following meetings/symposia during the past year:

1. Viruses of Microbes International Conference. Brussels, Belgium. Keynote symposium speaker. "X-ray Crystal Structure of PlyC, a Novel Enzybiotic" (July, 2012).

2. National Institute for Standards and Technology, Gaithersburg, MD. Invited Speaker for the Metrology of Microbial Systems Seminar Series. "Exploiting Bacteriophage Endolysins for Therapeutic and Diagnostic Use against Bacterial Pathogens" (November, 2011).
3. 3rd Annual Biomedical Sciences Day, The Universities at Shady Grove, Rockville, MD. "Evaluation of Bacteriophage Encoded Enzymes as Novel Therapeutic Agents against Biofilm-associated Infections" 1st Place Poster Presentation Award to Emilija Renke. (November, 2011)
4. University of Georgia, Athens, GA. Distinguished Alumni Marquee Speaker, Department of Biochemistry and Molecular Biology. "Structure/Function Studies on Enzybiotics" (October, 2011).
5. The 14th Annual Undergraduate Research Symposium in the Chemical and Biological Sciences. Baltimore, MD. "Utilization of Bacteriophage-Encoded Enzymes for the Vigorous Disruption of Biofilm Matrices" 1st Place Student Poster Award to Emilija Renke. (October, 2011)

**CONCLUSION:**

We have successfully analyzed the carbohydrate composition and linkage analysis of biofilm EPS for ESKAPE organisms and found that mannose is the predominant carbohydrate associated with the EPS. As such, we are investigating the anti-biofilm properties of mannosidases. In parallel, we have expressed and characterized ~15 putative depolymerase enzymes. Although there is species specific heterogeneity, several of these enzymes show broad anti-biofilm activity. these include DSPB, NagZ, EQ, and Betty. We consider these four enzymes our "lead compounds" at this point, although we continue to evaluate all 15 enzymes.

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**APPENDICES:** None.

**SUPPORTING DATA:**

Figures and figure legends are contained within the body of the text above.